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Adaptor protein sorting nexin 17 interacts with the scavenger receptor FEEL-1/stabilin-1 and modulates its expression on the cell surface

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ABSTRACT

The scavenger receptor FEEL-1/stabilin-1 is known as the marker of alternatively activated macrophage and sinusoidal endothelial cell. FEEL-1/stabilin-1 is a multifunctional transmembrane glycoprotein that is implicated in bacterial infection, diabetes, atherosclerosis, wound healing, and innate immunity. In the current study, we have identified the phox-homology domain containing protein SNX17 as a novel interaction partner of FEEL-1/stabilin-1 in endothelial cells. SNX17 directly interacts with FEEL-1/stabilin-1 and regulates its trafficking. Studies using the cytoplasmic domain of truncated or mutant FEEL-1/stabilin-1 suggest that the NPxF motif of the FEEL-1/stabilin-1 cytoplasmic tail is required for its interaction with SNX17. By transfecting cells with small interfering RNA targeting SNX17, total cellular FEEL-1/stabilin-1 expression and FEEL-1/stabilin-1-mediated ligand uptake were significantly decreased due to the enhancement of FEEL-1/stabilin-1 protein degradation. Our results identify SNX17 as a novel interaction partner of FEEL-1/stabilin-1 in endothelial cells.

1. Introduction

FEEL-1 and FEEL-2, which are also known as stabilin-1 and stabilin-2, respectively, are a novel family (type H) of scavenger receptors that are expressed in endothelial cells [1,2]. Stabilin-1 was initially identified as an MS-1 antigen expressed in sinusoidal endothelial cells in the human spleen [3,4] whereas stabilin-2 was detected as a hyaluronan-binding protein [5–7]. They are type I transmembrane proteins that contain a short cytoplasmic tail (CT), a transmembrane region, and a large extracellular region containing seven fasciclin domains, multiple epidermal growth factor-like domains, and a single C-type lectin-like hyaluronan-binding link domain [1,8]. Both FEEL-1/stabilin-1 and FEEL-2/stabilin-2 have predominant localization in EEA-1 (early endosomal antigen 1) positive endosomes, and both proteins can be detected on the surface of cells. There is no clear difference on the amount of FEEL-1/stabilin-1 and FEEL-2/stabilin-2 on the cell surface. [9–11].

FEEL-1/stabilin-1 is a multiligand endocytic receptor that recognizes various negatively charged macromolecules such as Gram-positive and Gram-negative bacteria; modified low-density lipoproteins (LDLs), i.e., acetylated LDL (Ac-LDL) and oxidized-LDL (Ox-LDL) [1]; advanced glycation end products (AGE) [12]; secreted protein acidic and rich in cysteine (SPARC) [13]; heat shock protein 70 (Hsp 70) [14]; and placental lactogen (PL) [15]. These ligands are implicated in bacterial infection, diabetes, atherosclerosis, wound healing, innate immunity, fetal growth, and placental angiogenesis, suggesting the pathophysiological significance of FEEL-1/stabilin-1 [2].

It has been reported that the CT of FEEL-1/stabilin-1 plays roles in two steps of its intracellular trafficking: (1) receptor-mediated endocytosis and recycling and (2) shuttling between the endosomal compartment and the trans-Golgi network (TGN) [16]. The above mentioned intracellular trafficking is mediated by Golgi-localized, γ-ear-containing 5′-diphosphate-ribosylation factor-binding adaptors (GGA), which interact with the DDSLL motif of the CT in alternatively activated human macrophages [9,11]. FEEL-1/stabilin-1 has also shown to associate with clathrin and adaptor protein-2 (AP-2) in rat liver sinusoidal endothelial cells [9]. Nevertheless, the mechanisms involved in FEEL-1/stabilin-1 endocytosis and recycling are still unknown.

The sorting nexins (SNXs) are a family of proteins involved in intracellular membrane trafficking that are characterized by the presence of a phox-homology (PX) domain, which is thought to play a

Abbreviations: Ac-LDL, acetylated LDL; AP-2, adaptor protein-2; APC, antigen-presenting cells; CMV, cytomegalovirus; CT, cytoplasmic tail; DiI, 1:1′-dioctadecyl-3:3:3′:3′-tetramethyl-indocarbo-cyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FERM, protein 4.1, ezrin, radixin, and moesin; GST, glutathione-S-transferase; HDL, high-density lipoprotein; Hsp70, heat shock protein 70; HUVEC, human umbilical vein endothelial cell; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; MHC, major histocompatibility complex; miRNA, microRNA; Ox-LDL, oxidized-LDL; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PL, placental lactogen; PX, phox-homology; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel for electrophoresis; SNX, sorting nexin; SPARC, secreted protein acidic and rich in cysteine; siRNA, small interfering RNA; TGN, trans-Golgi network

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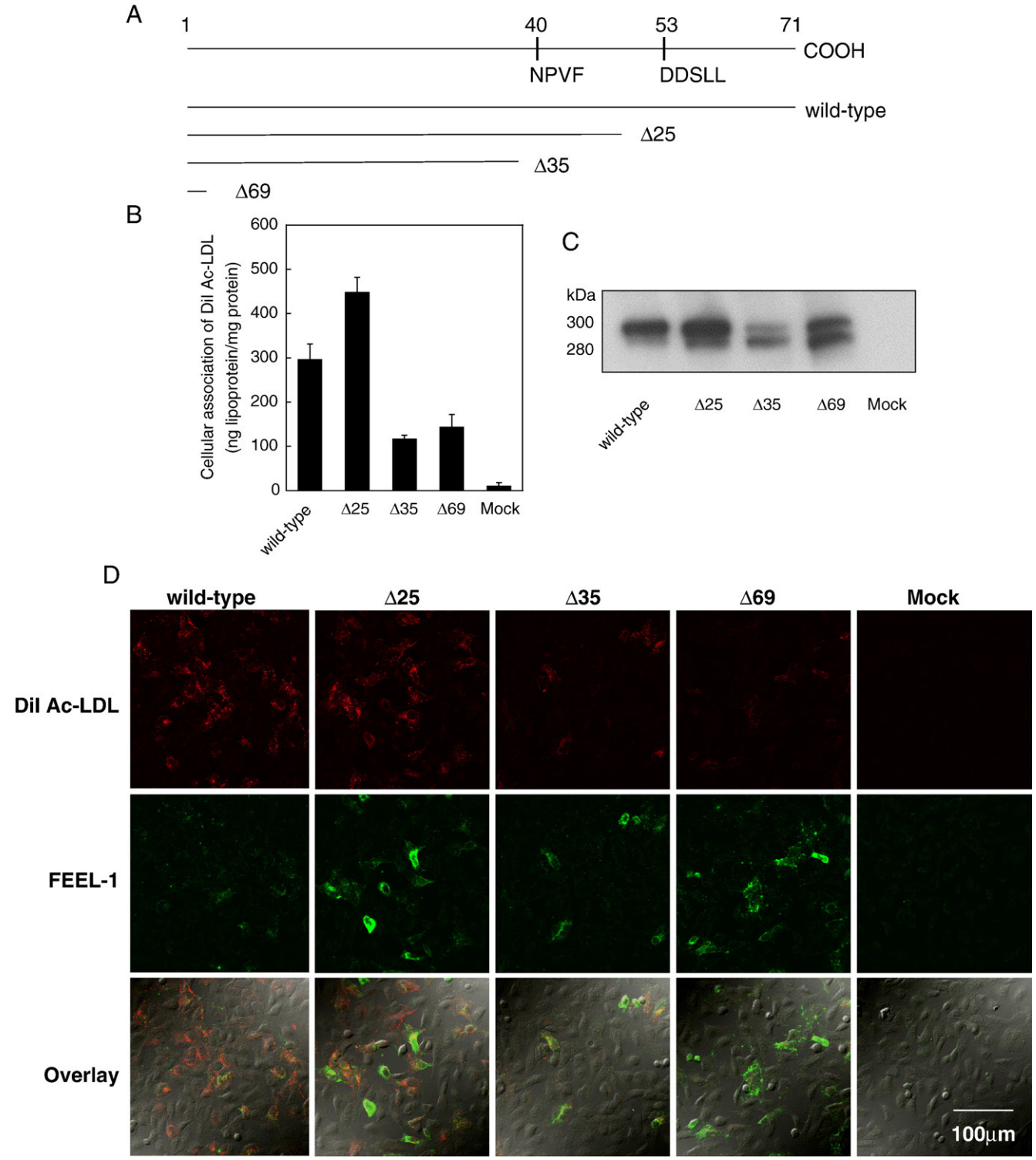

Fig. 1. Receptor activities of the cytoplasmic tail deletion mutant of FEEL-1/stabilin-1. (A) A schematic drawing of the cytoplasmic tail of FEEL-1/stabilin-1. Residue number 1 is the arginine residue 2500 following the putative transmembrane domain [1]. Deleted constructs and the positions of the NPVF and DDSLL domains are depicted. (B) The receptor activity of cytoplasmic tail mutants of FEEL-1/stabilin-1 transiently expressed in 293A cells. 0.6 μg of plasmid was transfected with Lipofectamine 2000 (Invitrogen) into 1×10^5 of 293A cells in a 24-well plate. After 48 h of transfection, the cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h at 37 °C. After washing, the cells were solubilized, and the cellular association of DiI Ac-LDL was determined fluorescently [1]. Data are expressed as mean ± SD. (C) Western blot analysis of truncated FEEL-1/stabilin-1. 293A cell lysate that expressed truncated FEEL-1/stabilin-1 was analyzed using monoclonal antibody to FEEL-1/stabilin-1 that recognizes its extracellular domain [1]. (D) Confocal microscopy of 293A cells transiently expressing wild-type or cytoplasmic tail mutant of FEEL-1/stabilin-1. 293A cells were seeded into 24-well plate and transfected with cDNA for wild-type or cytoplasmic tail mutants of FEEL-1/stabilin-1. The next day after transfection, cells were trypsinized and seeded into wells containing sterilized coverslips. After 48 h of transfection, cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. The expression of FEEL-1/stabilin-1 was detected by monoclonal antibody that recognizes its extracellular domain, using TSA Biotin System (NEN Life Science Products, MA, USA) and streptavidin conjugate Alexa Fluor 488 (Invitrogen).

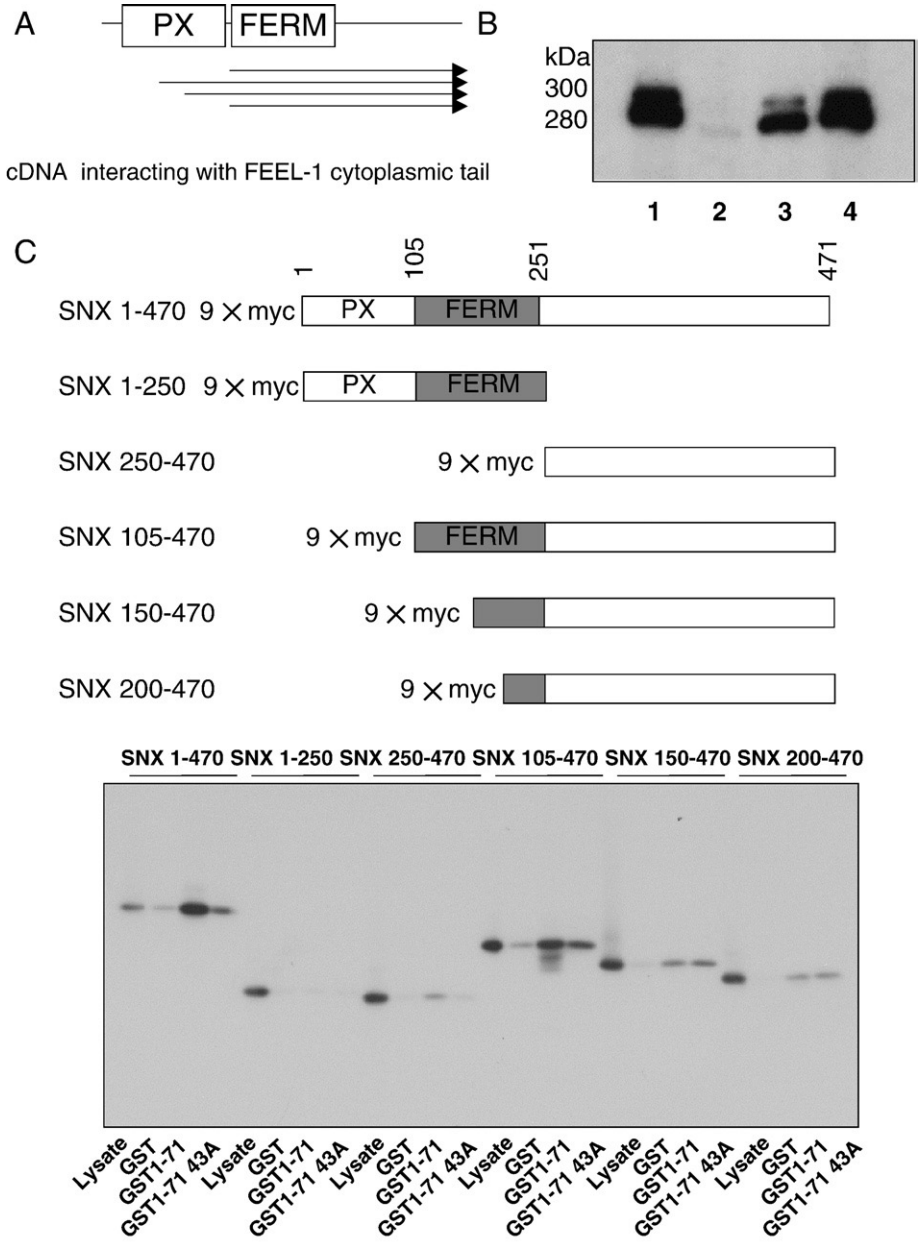

Fig. 2. Interaction of SNX17 with FEEL-1/stabilin-1. (A) Schematic drawing of SNX17 and cDNA interacting with the FEEL-1/stabilin-1 cytoplasmic tail. PX and FERM are depicted as phox-homology (PX) domain and a protein 4.1, ezrin, radixin, and moesin (FERM) domain. The arrows depict cDNAs interacting with the FEEL-1/stabilin-1 cytoplasmic tail obtained after yeast two-hybrid screening. (B) Interaction of SNX17 with FEEL-1/stabilin-1. 293A cell lysate that expressed both SNX17 and FEEL-1/stabilin-1 was immunoprecipitated with SNX17 antibody, and western blot was detected using anti FEEL-1/stabilin-1 (lane 1, lysate; lane 2, immunoprecipitated without antibody; lane 3, 0.2 μg of anti SNX17; lane 4, 0.4 μg of anti SNX17). (C) Schematic representation of 9 × myc-SNX17 and truncated constructs and myc-SNX17 pull-down assay by FEEL-1/stabilin-1cytoplasmic tail. GST or GST FEEL-1/stabilin-1 tail fusion (GST 1–71) or with a mutation in the SNX17-binding motif (GST 1–71 43A) were immobilized on glutathione-agarose beads and incubated with 293A cell lysate of myc-SNX17 truncated constructs. Bound proteins were analyzed by western blot using anti-myc antibody.

role in targeting the proteins to specialized membranes enriched in specific phospholipids [17]. SNX17 is a member of the SNX family that possesses a PX domain followed by a protein 4.1, ezrin, radixin, and moesin (FERM) domain [18]. The FERM domain and C-terminal region of the proteins are considered to be involved in the binding of their partners. Like many SNXs, SNX17 is localized in early endosomal vacuoles and recycling tubules and has been shown to interact with several proteins, such as LDL receptor, LDL receptor-related protein (LRP), P-selectin and amyloid precursor protein and modulate their endocytosis and recycling processes [19–21].

To elucidate the mechanism underlying the intracellular trafficking of FEEL-1/stabilin-1, we sought to find the FEEL-1/stabilin-1-interacting molecule that participates in receptor trafficking. In this study, we demonstrate for the first time that SNX17 binds to the CT of FEEL-1/stabilin-1. Our results suggest that SNX17 interacts with FEEL-1/stabilin-1 and thus modifies its intracellular recycling and degradation in endothelial cells.

2. Materials and methods

2.1. Plasmid constructs

For the expression of CT-deleted mutants in 293A cells, stop codons were introduced into the CT by the polymerase chain reaction (PCR) using plasmid DNA (pcDNA3-FEEL-1/stabilin-1) as a template [1]. pLexA (Clontech, Mountain View, CA, USA) was used for the expression of bait proteins in yeast. The cDNA of the whole CT (amino acids 2473–2570 of human FEEL-1/stabilin-1) containing the transmembrane portion of the

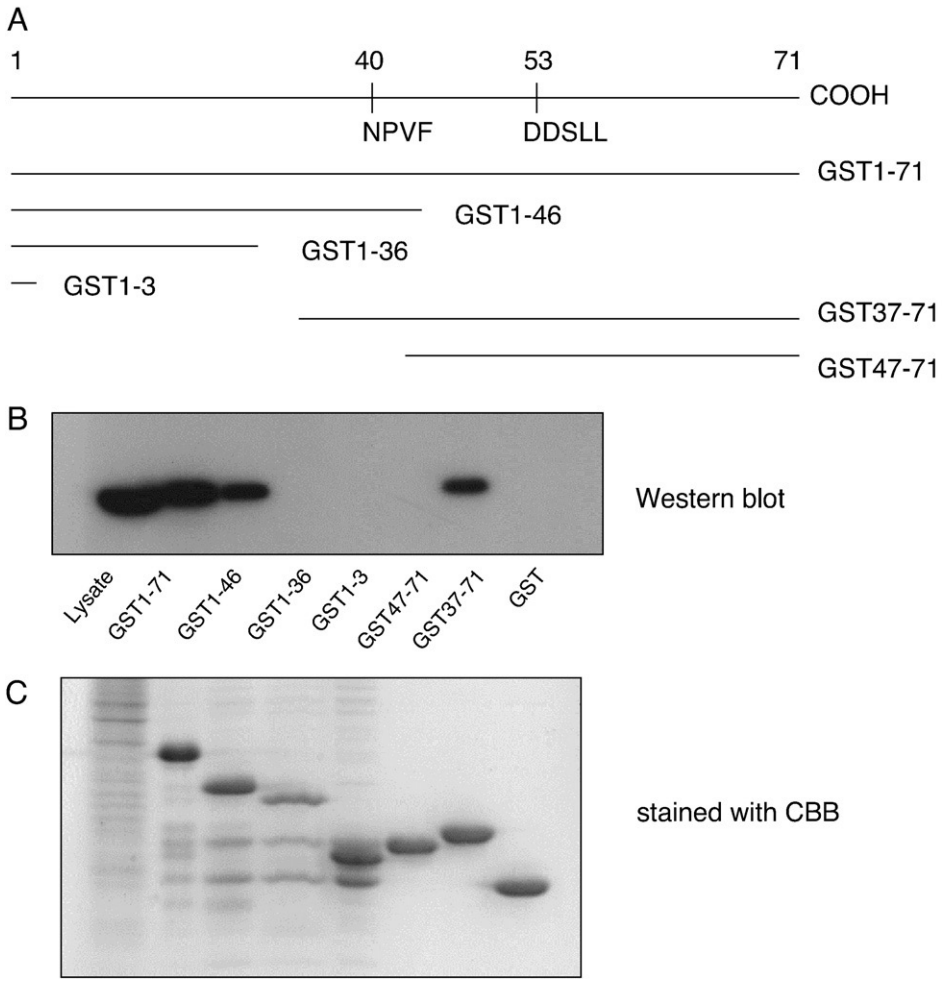

Fig. 3. The importance of the NPVF motif-containing sequence of FEEL-1/stabilin-1 in the interaction with SNX17. (A) A schematic drawing of truncated FEEL-1/stabilin-1 cytoplasmic tail mutants fused to glutathione-S-transferase (GST). Residue number 1 is the arginine residue 2500 following the putative transmembrane domain [1]. (B) Analysis of a pull-down assay by western blotting. Lysate containing SNX17 transiently expressed in 293A cells was incubated with cytoplasmic tail mutants fused to GST, which were bound to glutathione-Sepharose. After washing the beads, the bound proteins were separated by SDS-PAGE. Lane indicates the cytoplasmic tail mutants fused to GST or intact GST. After transferring the proteins to PVDF membrane, SNX17 was detected using anti-SNX17 antibody. (C) The gels were also stained with Coomassie Brilliant Blue R-250 as loading control.

FEEL-1/stabilin-1 molecule was amplified by PCR and cloned as an EcoRI–NotI fragment into the pLexA EcoRI and NotI sites downstream of the LexA sequence to produce pLexA-FEEL-1/CT. For mammalian expression, full-length SNX17 cDNA was obtained from Open Biosystems (Huntsville, AL, USA). For plasmids encoding GST fused to the cytoplasmic domain of FEEL-1/stabilin-1, appropriate fragments were amplified by PCR and cloned into pGEX-4T1 (GE Healthcare UK, Chalfont, St. Giles, UK). The point mutations were introduced into the CT of FEEL-1/stabilin-1 by inverse PCR using a KOD -Plus- Mutagenesis Kit (Toyobo, Osaka, Japan). 9× myc-tagged SNX17 was constructed by PCR using pTN184 (pBS-myc in XbaI(KS)) (National Bio Resource Project, NBRP, Japan) and full-length SNX17. 9× myc-tagged SNX17 domain constructs were constructed by inverse PCR using a KOD -Plus- Mutagenesis Kit (Toyobo, Osaka, Japan). All constructs were verified by nucleotide sequencing.

2.2. Yeast two-hybrid screening

Saccharomyces cerevisiae EGY48[p8oplacZ] was grown and used as the host for yeast two-hybrid screening as described by the supplier (Yeast Protocols Handbook, Clontech) The human umbilical vein endothelial cell (HUVEC) cDNA library was constructed in a pB42AD vector. After the simultaneous introduction of pLexA-FEEL-1/CT and the HUVEC cDNA library into *S. cerevisiae* EGY48[p8oplacZ], 2.4×10^6 cotransformants were selected and amplified on SD/-His/-Trp/-Ura plates. Cotransformants (1.0×10^7) were selected on induction medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to screen for the expression of *lacZ* and *LEU2* reporter genes. The cDNA from selected clones was amplified by PCR and characterized further by nucleotide sequencing.

2.3. Physical interaction of SNX17 with the cytoplasmic domain of the FEEL-1/stabilin-1

GST-CT fusion proteins were bound to glutathione *S*-Sepharose beads according to the supplier's instructions (Bulk GST Purification Module, GE Healthcare UK). SNX17 protein was produced in 293A cells (Invitrogen, Carlsbad, CA, USA) from a pCMV SPORT6 construct of human SNX17. The 293A cell lysate was prepared using Tris-buffered saline containing 0.25% (v/v) NP-40 (buffer A) and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Mannheim, Germany). Then, the 293A cell lysate (200 μl) was incubated with GST-fusion protein bound to glutathione-Sepharose beads (approximately 40 μl of a 50% slurry of beads) in buffer A at 4 °C for 3 h on a rotating wheel. The beads were washed three times with the same buffer, resuspended in 30 μl of sodium dodecyl sulphate (SDS) gel sample buffer containing 40 mM dithiothreitol (DTT), and aliquots were loaded onto gels of 8% SDS-polyacrylamide gels for electrophoresis. The gels were analyzed by western blot analysis using anti SNX17 antibodies. The gels were also stained with Coomassie Brilliant Blue R-250 as loading control.

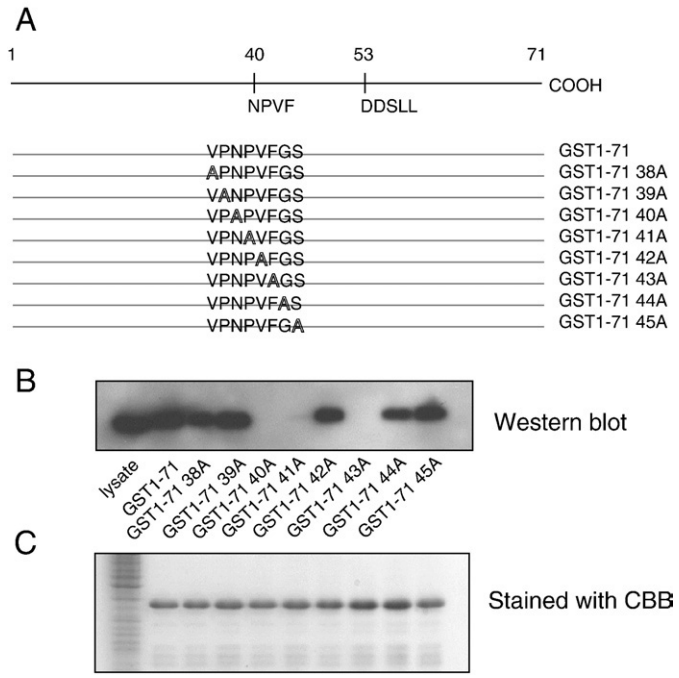

Fig. 4. The NPxF motif of the FEEL-1/stabilin-1 cytoplasmic tail is responsible for its interaction with SNX17. (A) A schematic drawing of FEEL-1/stabilin-1 cytoplasmic tail mutants. Residue number 1 is the arginine residue 2500 following the putative transmembrane domain [1]. (B) Analysis of a pull-down assay by western blotting. Lysate containing SNX17 transiently expressed in 293A cells was incubated with a cytoplasmic tail mutant fused to glutathione-S-transferase (GST), which was bound to glutathione-Sepharose. SNX17 was detected as described in Materials and methods. (C) The gels were also stained with Coomassie Brilliant Blue R-250 as loading control.

293A cells transfected with cDNA for FEEL-1/stabilin-1 protein or HUVECs were solubilized with buffer A and protease inhibitors, and soluble cell lysate were immunoprecipitated with anti SNX17 antibody. The beads were washed three times with the same buffer, resuspended in 30 μl of SDS gel sample buffer containing 40 mM DTT, and aliquots were loaded onto gels of 6% SDS-polyacrylamide gels for electrophoresis. The gels were analyzed by western blot analysis using anti FEEL-1 antibodies.

The different 9×myc-tagged SNX17 domain constructs were expressed in 293A cells and solubilized with buffer A and protease inhibitors. Then the lysate were incubated with glutathione-agarose beads that had bound with GST alone or GST-FEEL-1/stabilin-1 cytoplasmic domain without (GST 1–71) or with a mutation in the SNX17 binding motif (GST 1–71 43A). The beads were washed three times with the same buffer, resuspended in 30 μl of SDS gel sample buffer containing 40 mM DTT, and aliquots were loaded onto gels of 8% SDS-polyacrylamide gels for electrophoresis. The gels were analyzed by western blot analysis using anti-myc antibody (Nacali Tesque, Kyoto, Japan).

2.4. Detection of SNX17

A commercial rabbit antiserum (Proteintech Group, Inc. Chicago, IL, USA) or goat antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to detect the SNX17 protein. Anti-FEEL-1 antisera, which recognizes the CT of FEEL-1/stabilin-1, was used for immunological detection as described previously [1]. For the detection of cell surface truncated mutants of FEEL-1/stabilin-1 on 293A cells or FEEL-1/stabilin-1 on HUVECs, monoclonal antibody to FEEL-1/stabilin-1 were used [1].

2.5. Small interfering RNA (siRNA) transfection

siRNA was synthesized as Stealth™ RNAi (Invitrogen) and Stealth™ RNAi Negative Control Medium GC Duplex was purchased from Invitrogen. The nucleotide sequences were AACUGUUGAAAGUCUCG-CUGCUCC (siRNA SNX17(I)) or UUGAGAGUUUGACCAUAGACUCCCG (siRNA SNX17(II)) for SNX17 and AUGAGACAGCUGUUAAUUUCCUGGC (siRNA FEEL-1(I)) or AUCCUCCAUUGUUCUUAGAGCAGGG (siRNA FEEL-1(II)) for FEEL-1/stabilin-1. The 293A cells (1×10^5 cells/24-well plate) were cotransfected with pcDNA3/FEEL-1 and siRNA for SNX17 or control siRNA using Lipofectamine 2000 (Invitrogen) as recommended by the supplier. After the transfected cells had been cultured for a further 48 h, they were analyzed as follows: The cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h and solubilized with 1% Triton X-100 containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics). Then, the fluorescence intensity of the lysate was measured using a fluorescence multiple plate reader (CytoFluorII, PerSeptive Biosystems) [1].

2.6. Biotinylated FEEL-1 degradation

293A cells were cotransfected with pcDNA3/FEEL-1 and siRNA for SNX17 or control siRNA as described above. The cells were washed with cold phosphate-buffered saline (PBS), and cell surface proteins were labeled with EZ-Link Sulfo-NHS-SS-Biotin (1 mg/ml) (Pierce, Rockford, IL, USA) in cold PBS at 4 °C for 30 min. The reaction was terminated by washing and incubating the cells in PBS containing 100 mM glycine at 4 °C for 10 min. The cells were then incubated at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for the indicated periods. After solubilization of the cells with buffer A, which contained protease inhibitors, biotinylated proteins were precipitated from the lysate by using streptavidin-agarose (Sigma, Saint Louis, MO, USA) before being analyzed by western blotting. HiMark Pre-stained HMW Standard (Invitrogen) was run simultaneously to estimate the molecular weight of the protein on blots.

2.7. Cell surface expression assay—gene silencing by siRNA

HUVECs were transfected with RNAiMAX and stealth RNAi for 24 h. After transfection, the medium was changed, and the cells were cultured further for 24 h and analyzed as follows: The cells were incubated with 1 μg/ml of DiI (1:1′-dioctadecyl-3:3:3′:3′-tetramethyl-indocarbo-cyanine perchlorate) Ac-LDL for 3 h at 37 °C. After solubilization of the cells with 1% Triton X-100 containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics) the fluorescence intensity of the lysate was measured using a fluorescence multiple plate reader (CytoFluorII, PerSeptive Biosystems, Framingham, MA, USA) [1]. HUVECs were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (1 mg/ml) (Pierce, Rockford, IL, USA) in cold PBS at 4 °C for 30 min. The reaction was terminated by washing and incubating the cells in PBS containing 100 mM glycine at 4 °C for 10 min. After solubilization of the cells with buffer A, which contained protease inhibitors, biotinylated proteins were precipitated from the lysate by using streptavidin-agarose (Sigma, Saint Louis, MO, USA) before being analyzed by western blotting.

2.8. Confocal microscopy of cell surface expression of FEEL-1/stabilin-1

293A cells were seeded into 24-well plate and transfected with cDNA for wild-type or cytoplasmic tail mutants of FEEL-1/stabilin-1. The next day after transfection, cells were trypsinized and seeded into wells containing sterilized coverslips. After 48 h of transfection, cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. 293A cell clone that expresses wild-type or F2519A mutant of FEEL-1/stabilin-1 was seeded into wells containing sterilized coverslips. After 48 h of seeding, cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. HUVECs were seeded into wells containing sterilized

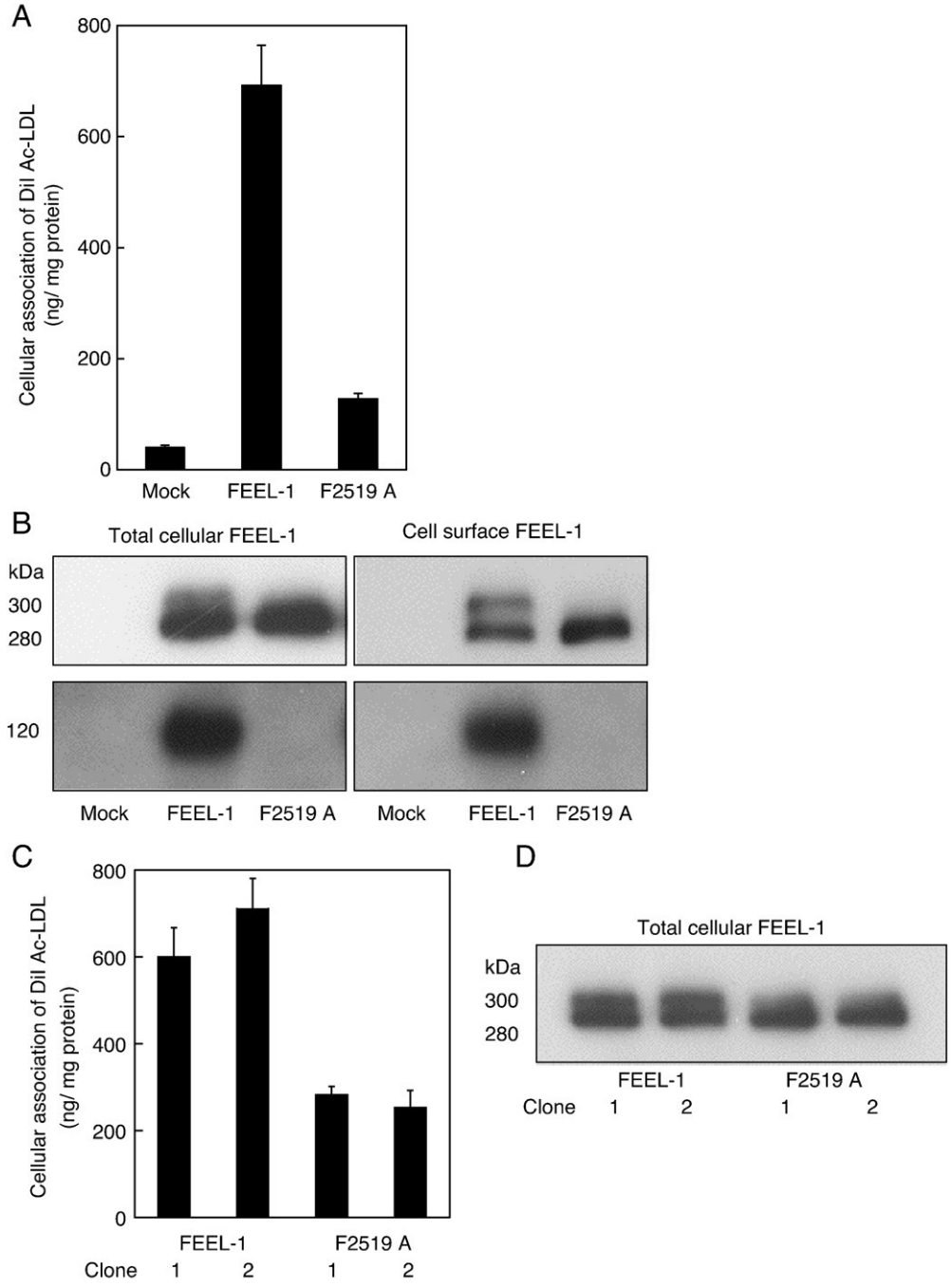

Fig. 5. Receptor activity and cellular distribution of wild-type and F2519A FEEL-1/stabilin-1. (A) The receptor activity of wild-type and F2519A FEEL-1/stabilin-1 transiently expressed in 293A cells. After 48 h of transfection, the cells were incubated with 1 μg/ml of DiI Ac-LDL for 2 h at 37 °C. After washing the cells, they were solubilized, and cellular association with DiI Ac-LDL was determined fluorescently [1]. Data are expressed as mean ± SD. (B) Quantification of cell surface expression and total cellular expression of wild-type and F2519A FEEL-1/stabilin-1 transiently expressed in 293A cells. After 48 h of transfection, cell surface proteins were labeled with biotin. The cells were then solubilized and then incubated with streptavidin-agarose. The bound biotinylated FEEL-1/stabilin-1 was detected by western blot analysis. Blots for the 120-kDa form were exposed for longer time. (C) The receptor activity of 293A clones that expressed wild-type or F2519A FEEL-1/stabilin-1. The cells were incubated with 1 μg/ml of DiI Ac-LDL for 2 h at 37 °C. After washing the cells, they were solubilized, and cellular association with DiI Ac-LDL was determined fluorescently [1]. (D) Quantification of total cellular expression of 293A clones that expressed wild-type or F2519A FEEL-1/stabilin-1.

coverslips and transfected siRNA using RNAiMAX. After 48 h of transfection, cells were incubated with 1 μg/ml of DiI Ac-LDL for 3 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. The expression of FEEL-1/stabilin-1 was detected by monoclonal antibody that recognizes its extracellular domain, using TSA Biotin System (NEN Life Science Products, MA, USA) and streptavidin conjugate Alexa Fluor 488 (Invitrogen). Confocal images were obtained using OLMPUS FV 1000-D confocal microscopy.

3. Results

3.1. Screening a HUVEC cDNA library by the yeast two-hybrid method for proteins interacting with the CT of FEEL-1/stabilin-1

To identify the cytoplasmic amino acid sequence of FEEL-1/stabilin-1 essential to its receptor activity, several cDNAs were prepared as deletion mutants and expressed in 293A cells. As

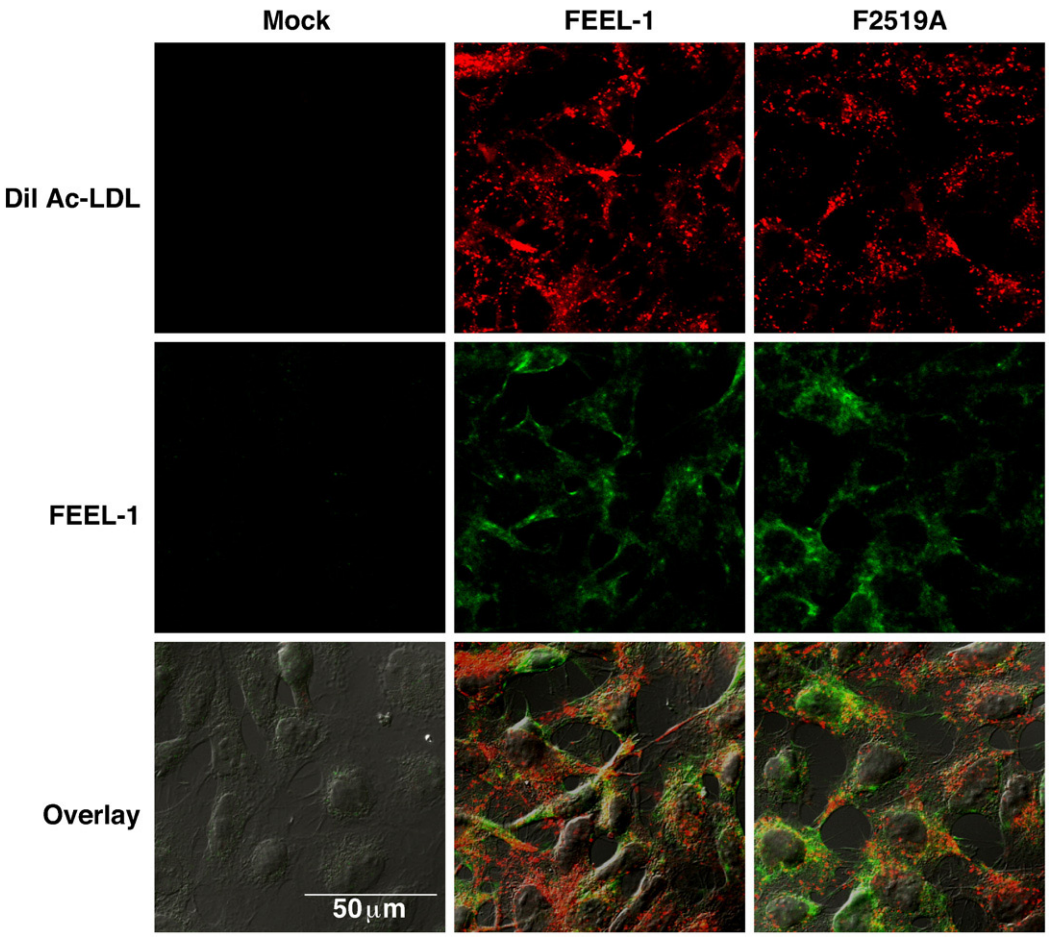

Fig. 6. Confocal microscopy of 293A cell clone that expresses wild-type or F2519A mutant of FEEL-1/stabilin-1. 293A cell clone that expresses wild-type or F2519A mutant of FEEL-1/stabilin-1 was seeded into wells containing sterilized coverslips. After 48 h of seeding, cells were incubated with 1 μg/ml of DiI Ac-LDL for 1 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. The expression of FEEL-1/stabilin-1 was detected by monoclonal antibody that recognizes its extracellular domain, using TSA Biotin System (NEN Life Science Products, MA, USA) and streptavidin conjugate Alexa Fluor 488 (Invitrogen).

shown in Fig. 1, the mock-transfected cells had little receptor activity, as measured by their cellular association with DiI Ac-LDL. As expected, transfection with full-length FEEL-1/stabilin-1 caused a significant increase in this activity. The cells transfected with a deletion mutant of the C-terminal 25 amino acids (Δ25) containing the DDSLL motif of the GGA adaptor protein binding site [10] showed increased activity, presumably due to increased steady-state expression (Fig. 1C). Further deletion of 10 (Δ35) or 44 (Δ69) amino acids from the CT significantly reduced receptor activity, suggesting that the sequence between 2535 and 2545 was important for such activity. Confocal microscopy of wild-type or cytoplasmic tail deletion mutants of FEEL-1/stabilin-1 showed that cells positive for FEEL-1/stabilin-1 were also positive for the intracellular DiI Ac-LDL containing endosomes (Fig. 1D).

Then, we searched for proteins that interacted with the amino acid sequence between 2535 and 2545 by two-hybrid yeast screening using the full-length cytoplasmic domain as bait. When 1.8×10^6 clones of a HUVEC cDNA library were screened with the bait, 8 clones showed reproducible interaction. Of these, four clones encoded a known protein, named sorting nexin-17 [18]. Fig. 2A shows the sequence of the SNX17 clones obtained by two-hybrid screening. Whereas all the sequences contained the entire FERM domain, two clones lacked the PX domain, suggesting that the PX domain was dispensable for the interaction between FEEL-1/stabilin-1 and SNX17. Then we examined whether SNX17 binds to FEEL-1/stabilin-1 in intact cells. 293A cells transfected with cDNA for FEEL-1/stabilin-1 were solubilized and soluble cell lysate was immunoprecipitated with anti SNX17 antibody. As shown in Fig. 2B, FEEL-1/stabilin-1 and SNX17 were co-immunoprecipitated with anti SNX17 antibody in a dose dependent manner. On the other hand, the receptor was barely detectable in a control precipitate when co-immunoprecipitation was performed in the absence of antibody, suggesting that the interaction indeed occurs in intact cells. Fig. 2C depicts the different 9× myc-tagged SNX17 domain constructs that were expressed in 293A cells. Cells extracts from transiently transfected 293A cells were incubated with GST alone or GST-FEEL-1/stabilin-1 cytoplasmic domain without (GST 1–71) or with a mutation in the SNX17 binding motif (GST 1–71 43A). The western blot in Fig. 2C shows that full-length SNX17 (SNX 1–470) interacted as expected, with the full-length FEEL-1/stabilin-1 cytoplasmic domain but weakly with F2519A mutant or GST alone. Both the N-terminal half (SNX 1–250) and C-terminal half of SNX17 (SNX 250–470) failed to interact with the FEEL-1/stabilin-1 cytoplasmic domain. Truncation of the N-terminal 105 (SNX 105–470) amino acid residues of SNX17 did not affect FEEL-1/stabilin-1 cytoplasmic domain binding. However, further truncation within FERM domain of SNX17 up to 150 amino acids (SNX 150–470) or 200 amino acids (SNX 200–470) resulted in a loss of FEEL-1/stabilin-1 cytoplasmic domain binding. These results suggested that the C-terminal region, but not PX domain of SNX17 contribute to FEEL-1/stabilin-1 cytoplasmic domain binding.

Direct physical interaction between SNX17 and the FEEL-1/stabilin-1 cytoplasmic domain was then confirmed by a pull-down assay using various FEEL-1/stabilin-1 CTs fused to glutathione-S-transferase (GST) that immobilized on glutathione *S*-Sepharose beads, and the lysate

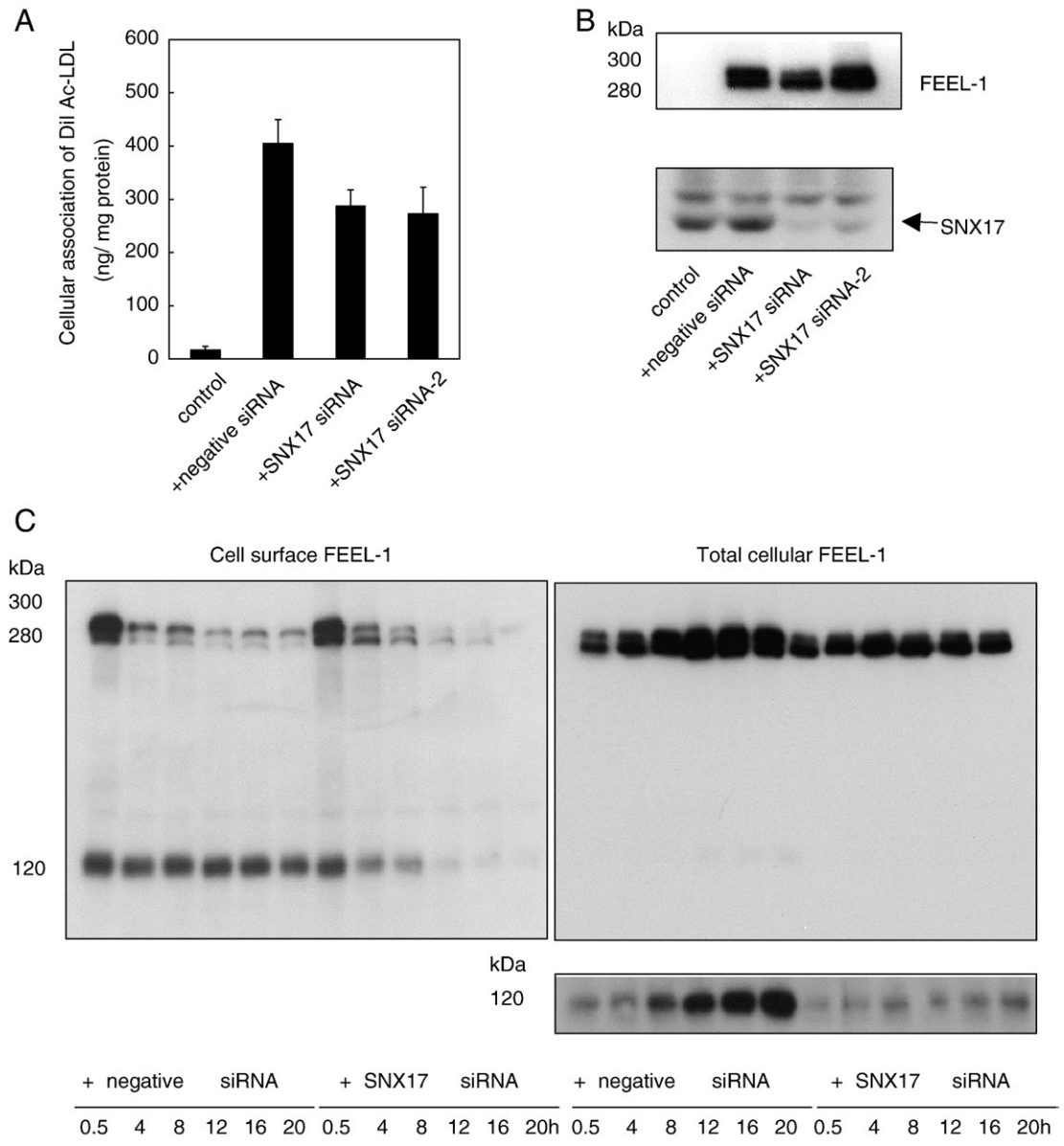

Fig. 7. Effects of siRNA treatment for SNX17 in 293A cells. (A) The receptor activity for DiI Ac-LDL in 293A cells that had been transfected with siRNAs for SNX17. 293A cells were cotransfected with FEEL-1/stabilin-1 and siRNA for SNX17 or negative control siRNA. After 48 h of transfection, the cellular association with DiI Ac-LDL was determined [1]. Data are expressed as mean ± SD. (B) Western blot analysis of FEEL-1/stabilin-1 and SNX17 expressed in 293A cells after siRNA transfection. (C) Degradation of FEEL-1/stabilin-1 in 293A cells treated with negative siRNA or SNX17 siRNA. 293A cells were cotransfected with FEEL-1/stabilin-1 and siRNA for SNX17 or negative control siRNA. After 48 h of transfection, cell surface proteins were labeled with biotin. The cells were then solubilized after the indicated period and then incubated with streptavidin-agarose. The bound biotinylated FEEL-1/stabilin-1 was detected by western blot analysis. Total FEEL-1/stabilin-1 was also shown by western blot analysis. 120 kDa form of total FEEL-1/stabilin-1 was shown in separate panel owing its low content in the total lysate.

prepared from 293A cells transiently expressed SNX17. As shown in Fig. 3, SNX17 bound to the GST-FEEL-1/CT 1–71 (full-length C-terminal cytoplasmic domain) and GST-FEEL-1/CT 1–46 mutants but not to the further deleted mutants GST-FEEL-1/CT 1–36, GST-FEEL-1/CT 1–3 or GST alone. Furthermore, SNX17 bound to GST-FEEL-1/CT 37–71 but not to GST-FEEL-1/CT 47–71. Taken together, these results suggest that FEEL-1/CT 37–47, which contains NPVF motif, was important for the interaction between FEEL-1/stabilin-1 and SNX17.

3.2. The NPVF motif in FEEL-1/stabilin-1 is required for its interaction with SNX17

To confirm the role of the NPVF motif in the interaction of FEEL-1/stabilin-1 with SNX17, we next replaced each residue around the motif with alanine, and the resulting GST-fusion proteins were incubated with the 293A cell lysate containing transiently expressed SNX17. As shown in Fig. 4, single point mutation of N 39, P 40, or F 42 to A resulted in a complete loss of the interaction with SNX17, indicating that these amino acid residues in the NPxF motif of the FEEL-1/stabilin-1 CT were essential for the interaction between SNX17 and the cytoplasmic domain of FEEL-1/stabilin-1.

3.3. SNX17 binding to FEEL-1/stabilin-1 is important for the cell surface expression and stability of FEEL-1/stabilin-1

To elucidate the significance of the interaction between FEEL-1/stabilin-1 and SNX17, wild-type and the F2519A (phenylalanine 2519 to alanine) mutant of FEEL-1/stabilin-1 were expressed in 293A cells, and the association of DiI Ac-LDL with these cells was compared (Fig. 5A). Expression of wild-type FEEL-1/stabilin-1 caused a significant increase

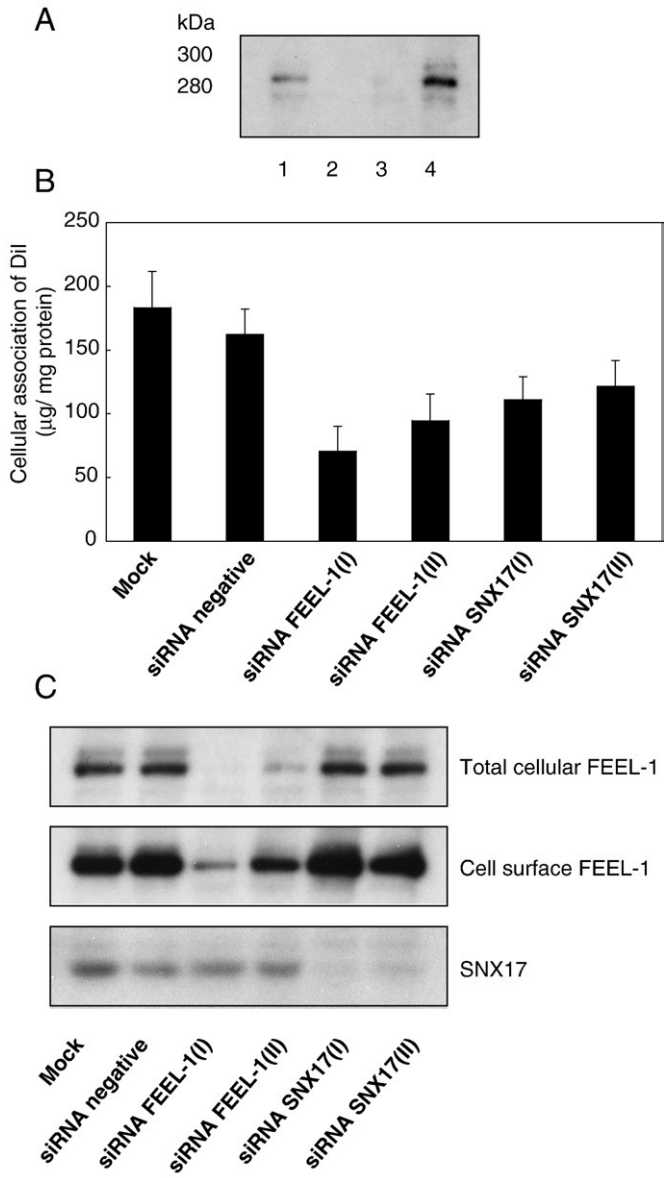

Fig. 8. Effects of siRNA treatment for SNX17 in HUVECs. (A) Interaction of SNX17 with FEEL-1/stabilin-1. HUVECs lysate were immunoprecipitated with SNX17 antibody, and western blot was detected using anti FEEL-1/stabilin-1 (lane 1, lysate; lane 2, immunoprecipitated without antibody; lane 3, 0.2 μg of anti SNX17; lane 4, 0.4 μg of anti SNX17). (B) The receptor activity for DiI Ac-LDL in HUVECs that had been transfected with siRNA for FEEL-1/stabilin-1 or SNX17 using RNAiMAX (Invitrogen). After 48 h of transfection, the cellular association of DiI Ac-LDL was determined fluorescently. Data are expressed as mean ± SD. (C) Western blot analysis of FEEL-1/stabilin-1 and SNX17 expressed in HUVECs after a 48 h siRNA transfection. Cell surface biotinylated FEEL-1/stabilin-1 was also analyzed by western blot.

in the cellular association of DiI Ac-LDL. On the contrary, when the FEEL-1/stabilin-1 CT mutant F2519A was expressed, the increase in the association was only marginal. To compare the expression of wild-type and mutant FEEL-1/stabilin-1 on the cell surface, biotinylation of cell surface proteins was performed. After solubilization, the biotinylated receptor was detected by western blot analysis (Fig. 5B). In agreement with Goerdt et al. [3], three forms (300, 280, and 120-kDa) of the wild-type receptor were detected on the cells. In contrast, only the 280-kDa form was detected when the mutant was expressed. These results suggested that the 280-kDa form of both wild and mutant FEEL-1/stabilin-1 were expressed on the cell surface independently from the interaction with SNX17 through the constitutive secretory vesicle. On the other hand, cell surface expression of 300-kDa and 120-kDa forms was apparently SNX17 dependent. Because the 120-kDa form was apparently observed only when the wild-type receptor was expressed, the 300-kDa form of the wild-type receptor might be cleaved proteolytically into 120-kDa during intracellular trafficking. Goerdt et al. [3] reported that the 300-kDa form is a mature form derived from the 280-kDa form. Further, to quantify the F2519A mutation, we generated cell lines stably expressing wild-type FEEL-1/stabilin-1 or F2519A mutant and selected clones that had similar expression level of FEEL-1/stabilin-1. Then we performed quantification of endocytosis of DiI Ac-LDL (Fig. 5C). F2519A mutant clones were rather inactive than wild-type FEEL-1/stabilin-1 clones as comparable to both receptors that transiently expressed in 293A cells. Western blot analysis of total cellular FEEL-1/stabilin-1 from these cell line suggested that F2519A mutant had low content of 300 kDa form and apparently accumulated 280 kDa form (Fig. 5D). Confocal microscopy of wild-type FEEL-1/stabilin-1 or F2519A mutant clones also suggested that cell surface expression of both FEEL-1/stabilin-1 were not significantly different because monoclonal antibody recognized both 280 and 300 kDa form of FEEL-1/stabilin-1 (Fig. 6). It also suggested that F2519A mutant was decreased in internalized DiI Ac-LDL rather than cell surface DiI Ac-LDL binding.

Then the stability of cell surface FEEL-1/stabilin-1 was determined using 293A cells that had been transfected with siRNA for SNX17 or with negative control siRNA. Cotransfection of siRNAs for SNX17 with FEEL-1/stabilin-1 reduced both the receptor activity (Fig. 7A) and the expression levels of endogenously expressed SNX17 and transiently expressed FEEL-1/stabilin-1, especially the 300-kDa mature form in cells (Fig. 7B). After transfection of siRNAs for SNX17, the endocytosis activity for Alexa-488 labeled transferrin was nearly the same as untreated intact cells (data not shown). After biotinylation of cell surface proteins, followed by chase incubation, the biotinylated FEEL-1/stabilin-1 was sedimented with streptavidin-agarose, and the amount of biotinylated FEEL-1/stabilin-1 was detected by western blot analysis. As shown in Fig. 7C, while the surface-labeled FEEL-1/stabilin-1 expressed in cells with SNX17 siRNA was degraded almost completely within 16 h, the negative siRNA-transfected control cells still contained a considerable amount of biotin-labeled 300-kDa mature and 120-kDa processed forms of FEEL-1/stabilin-1 for up to 20 h. As a result, 300-kDa and 120-kDa forms accumulated in total FEEL-1/stabilin-1. These results suggest that the interaction between SNX17 and the CT of FEEL-1/stabilin-1 delayed the degradation of the receptor.

Then we examined whether SNX17 binds to FEEL-1/stabilin-1 in HUVEC. HUVEC cells were solubilized and soluble cell lysate was immunoprecipitated with anti SNX17 antibody. As shown in Fig. 8A, FEEL-1/stabilin-1 and SNX17 were co-immunoprecipitated with anti SNX17 antibody in a dose dependent manner as in the case with 293A cells that transiently expressed FEEL-1/stabilin-1. To further confirm the significance of the interaction between FEEL-1/stabilin-1 and SNX17, two independent siRNAs for SNX17 were transfected into HUVECs. As shown in Fig. 8B, the cellular association of DiI Ac-LDL was reduced when siRNA for FEEL-1/stabilin-1 was expressed in HUVECs, as expected [1]. The expression of siRNA for SNX17 also caused reductions in the cellular association of DiI Ac-LDL. Notably, when siRNA for SNX17 was expressed in HUVECs, the expression levels of SNX17 were reduced and simultaneously accumulated cell surface 280-kDa form of FEEL-1/stabilin-1 (Fig. 8C), suggesting that reducing the expression of SNX17 caused a decrease in the expression of 300-kDa form of FEEL-1/stabilin-1 in HUVECs.

Internalization of DiI Ac-LDL and its colocalization of FEEL-1/stabilin-1 in HUVECs upon transfection of siRNAs were shown by confocal microscopy (Fig. 9). Transfection of siRNAs for FEEL-1 decreased its cell surface expression. Transfection of siRNA for SNX17 reduced cellular association of DiI Ac-LDL. As shown by the biotinylated cell surface FEEL-1/stabilin-1 in HUVECs (Fig. 8C), 280 kDa form of FEEL-1/stabilin-1 was apparently accumulated when HUVECs were transfected with siRNA for SNX17.

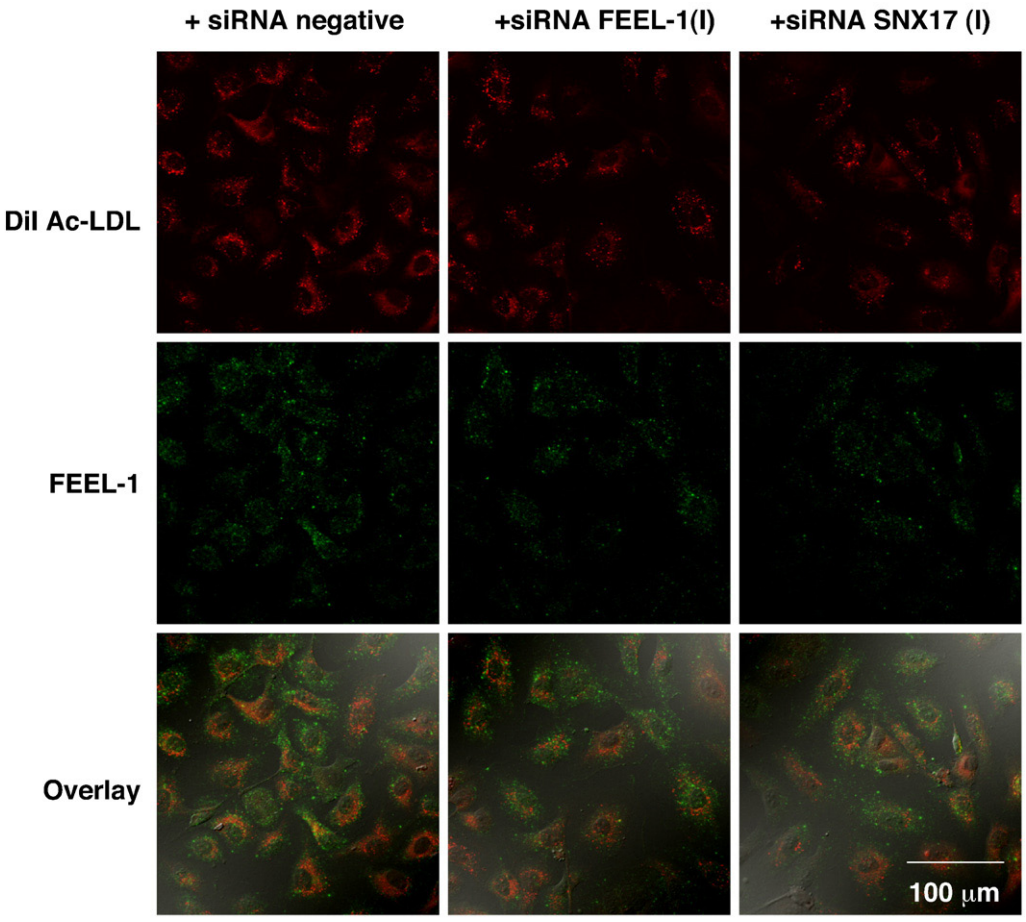

Fig. 9. Confocal microscopy showing siRNA effect on HUVECs. HUVECs were seeded into wells containing sterilized coverslips and transfected siRNA using RNAiMAX. After 48 h of transfection, cells were incubated with 1 μg/ml of DiI Ac-LDL for 3 h and then fixed in 3% (w/v) paraformaldehyde for 10 min. The expression of FEEL-1/stabilin-1 was detected by monoclonal antibody that recognizes its extracellular domain, using TSA Biotin System and streptavidin, conjugate Alexa Fluor 488.

4. Discussion

In this study, we demonstrated that SNX17 specifically recognized and bound to the NPxF motif of the FEEL-1/stabilin-1 cytoplasmic domain. Importantly, mutation of the NPxF motif caused a complete loss of the interaction and enhanced degradation of the receptor. It is conceivable that FEEL-1/stabilin-1 is initially transported to the plasma membrane as an inactive 280-kDa form in an SNX17-independent manner. After internalization, the inactive 280-kDa form binds to SNX17 and is recycled back to the plasma membrane as a mature 300-kDa active form. During recycling, the 300-kDa form might be cleaved proteolytically into a 120-kDa truncated form. In the absence of SNX17, the receptor is degraded rather rapidly in lysosomes. Taking all the data together into consideration, our results suggest that the interaction with SNX17 is required for the maximum cell surface expression of FEEL-1/stabilin-1.

It has been reported that SNX17 binds to the membrane-proximal NPVF motif of the LDL receptor CT and thus enhances the endocytosis and degradation of the receptor [20]. It has also shown that the membrane-proximal NPTY motif of LRP is required for interaction with SNX17 [19]. The loss of interaction causes decreased surface expression and enhanced lysosomal degradation of the receptor. On the other hand, the interaction of SNX17 with P-selectin inhibited the movement of P-selectin into lysosomes and thereby reduced its degradation [26]. Taken together, these results suggest that the interaction of SNX17 with NPxF/Y motif-containing receptors including FEEL-1/stabilin-1 affects intracellular trafficking and degradation both positively and negatively depending on the interaction partner and thus modulates their functions. However, we could not exclude the possibility that mutation of NPVF motif prevent the binding of other adaptor proteins to FEEL-1/stabilin-1.

The PX domain of SNX17 is known to bind to phosphatidylinositol-3 phosphate [19,23,24]. Treatment of 293A cells that transiently express FEEL-1/stabilin-1 with the PI-3K inhibitor wortmannin decreased receptor activity (data not shown), suggesting that the phosphatidylinositol-3-phosphate that is specifically distributed in recycling endosomes [22,25] is also important for FEEL-1/stabilin-1 recycling. PI-3K-dependent intracellular trafficking of scavenger receptor type IB (SR-BI) in hepatocytes has also been reported [26,30]. Decreased hepatocyte PI-3K activity in insulin-resistant states, such as type 2 diabetes, obesity, or metabolic syndrome, may impair reverse cholesterol transport by reducing the cell surface expression of SR-BI.

Although FEEL-1/stabilin-1 and FEEL-2/stabilin-2 share complex extracellular domains containing fasciclin domains, epidermal growth factor-like repeats, and C-type lectin-like hyaluronan-binding link modules, the cytoplasmic domains of the receptors have no obvious similarities [1,9]. Unlike FEEL-2/stabilin-2, the CT of FEEL-1 contains neither the classical YXXΦ (where Φ is a bulky hydrophobic residue) nor the NPXY consensus binding motif for AP-adaptor binding and clathrin-mediated endocytosis [9]. Instead, FEEL-1/stabilin-1 has a dileucine motif together with a cluster of acidic amino acids, which has been shown to bind to the GGA that mediates the traffic between the TGN and endosome/lysosomes [10]. The presence of the NPxF motif of the SNX17-binding site together with the GGA-binding site in FEEL-1/stabilin-1 (but not in FEEL-2/stabilin-2) may explain the differences in the intracellular localization of these receptors. While FEEL-1/stabilin-1 shows a largely intracellular localization, FEEL-2/stabilin-2, which contains the plasma membrane endosomal sorting motifs NPLY and

YDPE in its CT, is expressed in the plasma membrane [9]. After being transported from the TGN to endosomes, where SNX17 is localized, FEEL-1/stabilin-1 may bind to SNX17 and enter into the recycling pathway. In the absence of SNX17-binding, it is plausible that the lysosomal delivery of FEEL-1/stabilin-1 is enhanced through the change of its recycling rate in the cell, and thus, cell surface receptor activity is decreased. On the other hand, the decreases in SNX17 expression caused by siRNA treatment caused a decrease in 300 kDa for of FEEL-1/stabilin-1 expression, indicating that the interaction also affected the abundance of the receptor in cells. It was recently reported that the interaction of SR-BI with PDKZ1 also affected the expression and intracellular localization of SR-BI [27]. It is plausible that the pathophysiological functions of several receptors are regulated by their binding partners by changing their expression and intracellular trafficking levels in cells.

It is becoming evident that FEEL-1/stabilin-1 is multifunctional, and various ligands have been reported for it. Clearance of SPARC is mediated by FEEL-1/stabilin-1 in alternatively activated human macrophages [13]. The matricellular protein SPARC, also known as osteopontin, is implicated in development, differentiation, response to injury, and tumor suppression by virtue of its regulation of extracellular matrix production/assembly and its antiadhesive and antiproliferative effects on different cell types [28]. FEEL-1/stabilin-1 interacting chitinase-like protein (SI-CLP) is also shown to be a ligand for FEEL-1/stabilin-1. Research indicates that FEEL-1/stabilin-1 was involved in intracellular sorting of SI-CLP by binding with its extracellular domain [29]. The extracellular levels of PL are regulated by its FEEL-1/stabilin-1-mediated uptake and transcytosis in alternatively activated human macrophages [15]. Furthermore, we have reported that Hsp70 is also a ligand for FEEL-1/stabilin-1 [14]. After internalization of extracellular Hsp70 by antigen-presenting cells (APC), the complex is thought to be trafficked into the cytoplasm or endosomal vesicles before the release, processing, and representation of the immunogenic peptide cargo by major histocompatibility complex (MHC) class I and II molecules at the surface of APC [30]. Given that FEEL-1/stabilin-1 is an endocytic receptor for these ligand proteins, it is tempting to speculate that the receptor plays important roles in the regulation of the activities of its ligands by regulating their degradation rates. SNX17 might play roles in facilitating FEEL-1/stabilin-1 recycling in early endosomes and regulate the delivery of its ligands to lysosomes.

In conclusion, our findings demonstrate that SNX17 plays an important role in the intracellular trafficking and plasma membrane localization of FEEL-1/stabilin-1 in endothelial cells. SNX17 may take part in clearance of endocytosed ligands and thus regulate their pathophysiological activities.

Acknowledgements

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